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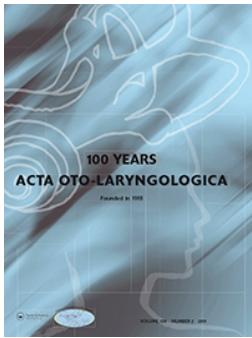
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RESEARCH ARTICLE



TLR5 and TLR7 are differentially expressed in human papillomavirus-positive and negative base of tongue squamous cell carcinoma, and TLR7 may have an independent prognostic influence

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ABSTRACT

Background: Human papillomavirus-positive (HPV⁺) base of tongue squamous cell carcinoma (BOTSCC) has a better outcome than corresponding HPV[−] cancer. TLR5 and TLR7 expression was previously shown to differ depending on HPV – status and correlate with outcome in oropharyngeal squamous cell carcinoma.

Aims/objectives: For validation, TLR5 and TLR7 were analyzed in a BOTSCC-cohort for correlation with HPV, survival, CD4⁺ and CD8⁺ tumor-infiltrating lymphocyte (TIL) counts, the latter being a well-documented prognostic marker.

Materials and methods: BOTSCC biopsies, (49HPV⁺/28HPV[−]) were analyzed by immunohistochemistry for TLR5 and TLR7, and correlated with the above parameters.

Results: TLR5 expression was more frequently absent/weak than medium/strong in HPV⁺ compared to HPV[−] BOTSCC ($p < .001$). The opposite was observed for TLR7 ($p < .007$). TLR5 and TLR7 expression did not correlate to survival in either the HPV[−] or HPV⁺ cases, or to CD4⁺ TILs. TLR5, (but not TLR7) expression was correlated to CD8⁺ TIL counts ($p = .023$).

Conclusion and significance: Absent/weak TLR5 and medium/strong TLR7 expression was validated as more frequent in HPV⁺ compared with HPV[−] BOTSCC. A correlation between CD8⁺ TIL counts, and TLR5 expression was disclosed, but not with TLR7. Therefore, it could be useful to investigate TLR7 further as a potential independent prognostic marker.

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KEYWORDS

HPV; base of tongue cancer; tonsillar cancer; oropharyngeal cancer; TLR 5; TLR7 immunohistochemistry; survival

Introduction

Patients with human papillomavirus-positive (HPV⁺) oropharyngeal squamous cell carcinoma (OPSCC) have a much more favorable clinical outcome than those with HPV negative (HPV[−]) OPSCC [1–3]. In the Western world the incidences of tonsillar and base of tongue squamous cell carcinoma (TSCC/BOTSCC), the OPSCC subtypes where HPV is most common, have increased [4–7]. Notably, treatment for head and neck squamous cell carcinoma include chemo-radiotherapy, targeted therapy, and surgery either alone or in combination. These treatment modalities expose the patients to risks of therapy-related side effects and complications. Most HPV⁺ TSCC/BOTSCC patients do not need intensified treatment, and to de-escalate treatment and reduce side effects, would be of benefit for most patients [3,8,9]. To identify patients who will respond readily to protocol treatment, attempts have been made to find additional predictive markers, e.g. age, stage, high CD8⁺ tumor-infiltrating lymphocyte (TIL) counts, HPV16 E2

mRNA expression, absent/weak CD44, or high LRIG1 or CD98 expression and use mathematical models [3,9–14]. In addition, we recently explored the impact of Toll-like receptors (TLR) in OPSCC and their influence on survival [15]. In that study tumor biopsies of 202 OPSCC diagnosed in Helsinki, Finland, were analyzed for the presence of TLR5, 7 and 9, and the expression of some of these TLRs was correlated with clinical outcome. TLR5, 7 and 9 expression differed between HPV⁺ and HPV[−] OPSCC, and in general HPV⁺ OPSCC exhibited lower TLR5 and higher TLR7 expression levels than HPV[−] OPSCC [15]. Furthermore, high expression of TLR5 and low expression of TLR7 correlated with worse disease-specific survival (DSS) in HPV⁺ OPSCC [15]. To validate the data in a new cohort in another Nordic population and in a specific OPSCC subsite, biopsies from 77 BOTSCC patients from Stockholm, Sweden, were analyzed for TLR5 and TLR7 expression and the data were correlated with clinical outcome. Since TLRs initiate immune responses [15–17], we also examined if

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TLR5 and/or TLR7 expression was correlated with CD4⁺ and/or CD8⁺ TIL counts in the tumors, because the presence of high CD8⁺ TIL counts is reported to be a good prognostic marker in HPV⁺ OPSCC [9,12,13].

Materials and methods

Patients and tumor characteristics

Patients diagnosed from 2000 to 2011 at the Karolinska University Hospital with BOTSCC (ICD-10 code C01.9), were analyzed for TLR5 and TLR7 expression by immunohistochemistry (IHC) in order to retrospectively compare TLR5 and TLR7 expression in patients with HPV⁺ and HPV⁻ tumors. Details of the patients and their tumors are presented in Table 1 together with *p* values. HPV⁺ BOTSCC was defined as having an HPV DNA positive tumor with p16^{INK4A} overexpression (p16⁺) [18]. In total, 49 HPV⁺ BOTSCCs and 28 HPV⁻ BOTSCCs fulfilling these criteria according to our previous studies [5,7,14] were analyzed for TLR5 and TLR7 expression [15]. The study was performed in compliance with permission (2009/1278-31/4) granted by the Regional Ethical Review Board in Stockholm.

Analysis of HPV DNA, p16 overexpression, and CD4⁺ and CD8⁺ TILs

All samples had been tested for HPV DNA by a PCR-based bead-based multiplex-assay on a MagPix instrument (Luminex Inc.) as described before [19]. p16 had been assayed using the monoclonal antibody (mAb) clone JC8 (Santa Cruz Biotech, Santa Cruz, CA), or the E6H4TM mouse mAb clone (CINtec®, Ventana, Tucson, AZ) and CD4⁺ and CD8⁺ with the mAb clone 1FC and 4B11 respectively, (Leica Biosystems, Wetzlar, Germany) [19].

Immunohistochemistry for TLR 5 and 7

TLR5 and 7 expression by IHC on 4 µm FFPE sections were investigated and has been reported before [15]. Monoclonal mouse anti-human TLR5 (1:200, IMG-664A Imgenex) and monoclonal rabbit anti-human TLR7 (1:300, IMG-581A Imgenex) were used. All tissue samples were evaluated separately for TLR5 and 7 expression by light microscopy, by two researchers (LH and AN), of which the latter is a resident in surgical pathology. The samples were evaluated independently, without the knowledge of clinical parameters. The scoring of the two researchers was similar in the majority of the cases, and when not, a consensus was reached. TLR5 intensity, mainly localized to the cell membranes, was scored as: absent (0), weak (1), medium (2), and strong (3). TLR7 intensity, mainly localized to the nuclear membrane and nucleus, was scored as absent (0) if no TLR7 was observed, weak (1) if some nuclear membranes were positive, medium (2) if all nuclear membranes and some nuclei were stained, and strong (3) if nuclear membranes and nuclei were stained substantially. Cases, where the staining was not possible to be evaluated, were excluded.

Statistical analysis

Categorical variables (e.g. TLR5 and TLR7 expression by IHC) were compared with the Chi2-test or Fisher's exact test, and continuous variables (CD8⁺ TIL counts) with Student's *t*-test. Clinical outcome was measured as 3-year disease-free survival (DFS) or 3-year disease-specific survival (DSS). Only patients treated with intent to cure were included in the analysis. All patients were followed up for 3-years and then censored. DFS was defined as the time from diagnosis until date of relapse in disease. Patients being never tumor-free were censored on day 0, while patients dying without prior recurrence were censored at the time-point, when assessing DFS. DSS was defined as the time from diagnosis until date of relapse or death with the disease. Survival curves with 3-year DFS and DSS were calculated using the Kaplan–Meier method and differences in survival were tested using the log-rank test. All statistical tests were performed using SPSS (SPSS Statistics for Mac, Version 25. Armonk, NY: IBM Corp.).

Results

Analysis of TLR5 and TLR7 expression by IHC in HPV⁺ and HPV⁻ BOTSCC

Totally, 49 HPV⁺ and 28 HPV⁻ BOTSCC samples were examined for the expression of TLR5 and TLR7 by IHC (Table 1). Absent/weak TLR5 staining was observed in 41/49 (84%) HPV⁺ BOTSCC and 12/28 (43%) of HPV⁻ BOTSCC (*p* < .0001). In contrast, TLR7 was more often absent/weak in HPV⁻ BOTSCC (26/28, 93%) as compared with HPV⁺ BOTSCC (32/49, 65%) (*p* = .007).

TLR5 and TLR7 expression, and CD8⁺ TILs, in relation to outcome in HPV⁺ and HPV⁻ BOTSCC

The presence of absent/weak TLR5 and TLR7 expression was then correlated with prognosis among the 68 patients in the whole cohort, and separately among the 47 patients with HPV⁺ BOTSCC, and the 21 patients with HPV⁻ BOTSCC, who had been treated with curative intent, although in the latter group the patient number was limited.

In all the examined cases, absent/weak TLR5 or TLR7 did not have a statistically significant correlation with clinical outcome defined as OS, DFS or DSS. For HPV⁺ BOTSCC, Kaplan–Meier graphs for DFS and DSS are depicted for absent/weak and moderate/high TLR5 expression, as well as for absent/weak and moderate/high TLR7 expression in Figure 1.

TLR5 and TLR7 expression in correlation to CD8⁺ and CD4⁺ TIL counts

The presence of TLR5 and TLR7 expression in correlation with CD4⁺ and CD8⁺ TIL counts was examined in all patients. Patients with absent/weak TLR5 expression had significantly higher CD8⁺ TIL counts (*p* = .023), but no correlation was found for CD4⁺ TIL counts (*p* = .17). In

Table 1. Patient and tumor characteristics.

Patient and tumor characteristics	HPV + BOTSCC(N = 49)		HPV + BOTSCC(N = 28)		p value
	N	%	N	%	
Age					
Mean (years)	62		62		
Median (years)	62		64		
Range (years)	42–82		32–85		
Sex					
Female	14	29%	10	36%	.971*
Male	35	71%	18	64%	
Tumor differentiation					
Poorly	36	73%	15	54%	.517§
Moderately	9	18%	11	39%	.055§
Well	3	6%	2	7%	(poorly vs. moderate/well)
Undefined	1	2%	0	0%	
T stage (Tumor size)					
T1	15	31%	5	18%	.005§
T2	17	35%	4	14%	(T1 & T2 vs. T3 & T4)
T3	3	6%	6	21%	
T4	14	29%	13	46%	
N stage (Nodal disease)					
N0	9	18%	10	36%	.739§
N1	10	20%	2	7%	(N0 & N1 vs. N2 & N3)
N2a	8	16%	2	7%	
N2b	11	22%	6	21%	
N2c	9	18%	6	21%	
N3	2	4%	2	7%	
M stage (Distant metastasis)					
M0	46	94%	27	96%	1¶
M1	2	4%	1	4%	(M0 vs. M1)
MX	1	2%	0	0%	
Tumor stage (AJCCs 8th edition)					
I	12	24%	3	11%	.001§
II	21	43%	5	18%	(stage I & II vs. stage III & VI)
III	14	29%	2	7%	
IV	1	2%	0	0%	
IVa	0	0%	16	57%	
IVb	0	0%	1	4%	
IVc	0	0%	1	4%	
Treatment					
Unknown	1	2%	0	0%	
Induction chemotherapy and radiation (conventional/accelerated)	22	45%	9	32%	.764§
Radiation only (conventional/accelerated)	25	51%	12	43%	(chemo/radio vs. radio)
None/surgery/palliative	2	4%	7	25%	<.0001§
Smoking					
Never	22	45%	5	18%	(never/>15 years ago vs. current/<15 years ago)
Former (>15 years ago)	11	22%	1	4%	
Former (<15 years ago)	11	22%	1	4%	
Current upon diagnosis	5	10%	21	75%	.0002§
TLR 5 membrane expression					
Absent	29	59%	8	29%	(absent/weak vs. medium/strong)
Weak	12	24%	4	14%	
Medium	3	6%	9	32%	
Strong	5	10%	7	25%	
TLR 7 nuclear membrane and nuclear expression					
Absent	18	37%	17	61%	.012§
Weak	14	29%	9	32%	(absent/weak vs. medium/strong)
Medium	13	27%	2	7%	
Strong	4	8%	0	0%	
CD8+ tumor infiltrating lymphocyte (TIL) count					.009*
Median (number of TILs)	39.7		8.1		
Range (number of TILs)	1.0–414.8		0.1–101.9		
CD4+ tumor infiltrating lymphocyte (TIL) count					.018*
Median (number of TILs)	16.7		8.5		
Range (number of TILs)	0.0–303.7		0.0–35.0		

*Two-tailed student's *t*-test, comparison of means.

§Chi-square test, Pearson.

¶Two-tailed Fisher's exact test.

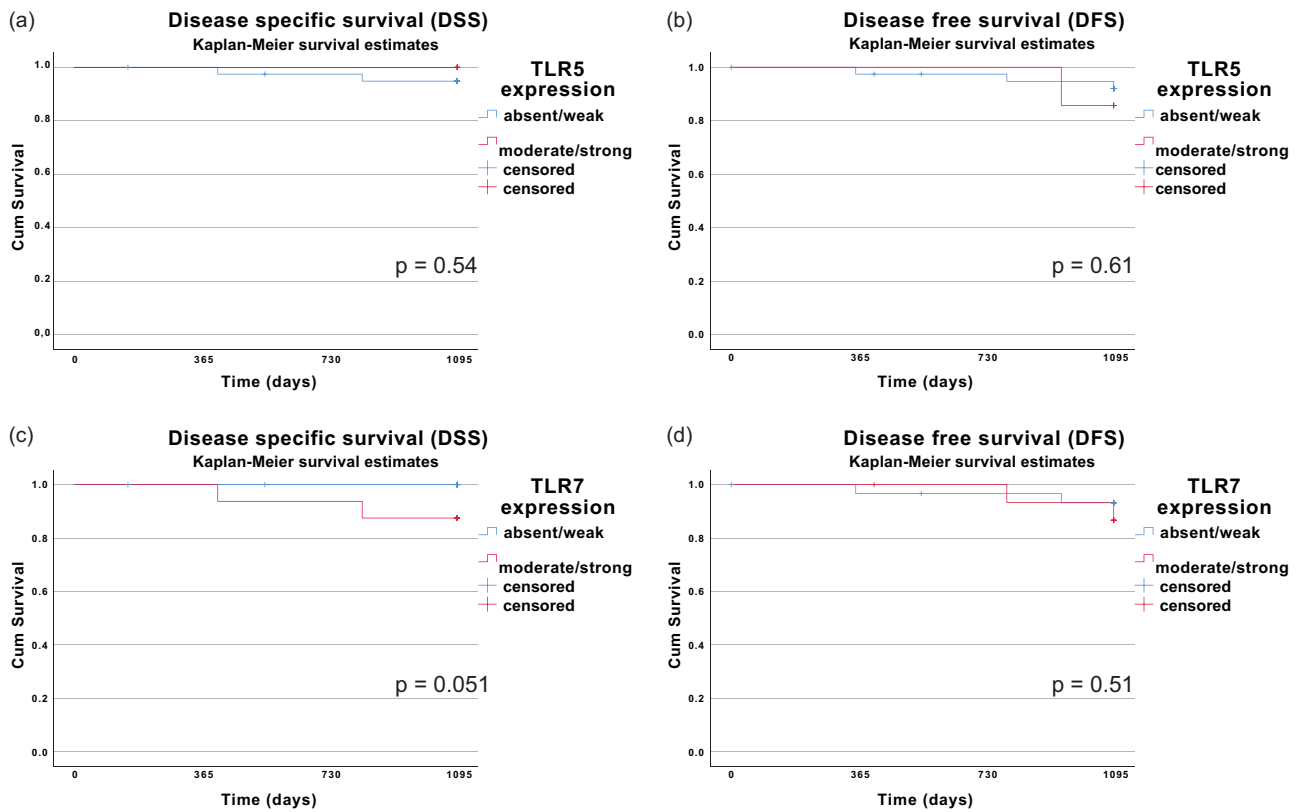


Figure 1. Kaplan-Meier curves with 3-year disease-specific survival (DSS) and 3-year disease-free survival (DFS) for patients with HPV⁺ BOTSCC stratified for absent/weak vs. medium/strong TLR5 expression (A and B) and absent/weak vs. medium/strong TLR7 expression (C and D). Differences in survival was analyzed with the log-rank test. No differences in survival were observed (*TLR5 DSS*: $p = .54$; *TLR5 DFS*: $p = .61$; *TLR7 DSS*: $p = .051$; *TLR7 DFS*: $p = .51$).

addition, for patients with HPV⁺ BOTSCC the correlation between absent/weak TLR5 expression and high CD8⁺ TIL counts remained ($p = .038$), while this was not the case for corresponding HPV⁻ BOTSCC cases $p = .92$. No correlation could be observed either between TLR7 expression and CD4⁺ TIL or CD8⁺ TIL counts ($p = .43$ and $p = .58$, respectively), or when dichotomizing the patients into those with HPV⁺ or HPV⁻ BOTSCC ($p = .17$ and $p = .94$, respectively).

Discussion

This study aimed to validate whether TLR5 and TLR7 expression differed between HPV⁺ and HPV⁻ BOTSCC (i.e. in a specific subtype of OPSCC), as has been previously shown in HPV⁺ and HPV⁻ OPSCC and whether TLR5 and TLR7 expression would correlate with survival in HPV⁺ BOTSCC [15]. Our results confirmed that absent/weak TLR5 and medium/strong TLR7 expression was more frequently found in HPV⁺ compared with HPV⁻ BOTSCC [15,20].

However, it was not possible to confirm with statistical significance that moderate/strong TLR5 or absent/weak TLR7 expression would correlate with worse outcome irrespective of tumor HPV status. TLR5 and TLR7 expression was also analyzed for correlation with CD4⁺ and CD8⁺ TIL counts and a statistically significant correlation between absent/weak TLR5 expression and the presence of high CD8⁺ TIL counts in all patients, as well as in patients with

HPV⁺ BOTSCC, was shown. There was, however, no correlation either between TLR7 expression and CD8⁺ TIL counts or between CD4⁺ TIL counts, and TLR5 and TLR7 expression.

Consequently, in this Scandinavian BOTSCC cohort, we confirmed similar results compared with the previous study in an OPSCC cohort [15] stating that the expression of TLR5 and TLR7 varied according to HPV status, but not that moderate/strong TLR5 and absent/weak TLR7 expression correlated with survival. In fact, the trend was the opposite for DSS and TLR7 expression. The reasons for the latter discrepancies in clinical outcome and TLR5 and TLR7 expression between this study and the aforementioned study on OPSCC are unknown. The most plausible explanation could be that in this study the number of events was limited due to the favorable outcome for patients with HPV⁺ BOTSCC. Furthermore, the limited size of the cohort had an obvious effect on possibilities to encounter such events [15]. Nevertheless, as discussed further below, the potential role of TLR7 expression on survival should be studied further in a larger HPV⁺ BOTSCC cohort, and if possible with proteomic and transcriptomic analysis.

For HPV⁻ BOTSCC no correlation between TLR5 and TLR7 expression was found, similarly to previously reported results [15]. This could, however, be due to that in our study the number of HPV⁻ cases were limited.

In this report, TLR5 and TLR7 expression was also investigated for possible correlation with the presence of CD4⁺ and CD8⁺ TIL counts since TLRs recognize molecular

patterns, which can initiate immunological defense mechanisms against bacteria and viruses [17]. High CD8⁺ TIL counts correlated with absent/weak TLR5 expression. This finding was not entirely unexpected since both absent/weak TLR5 expression, as well as high CD8⁺ TIL counts, are correlated with the presence of HPV [11,15].

There are some limitations in this study. Here, only BOTSCC samples were examined and no TSCC cases were included. More importantly, this gave us an opportunity to really focus on one tumor subtype and clearly, the association between absent/weak TLR5 and medium/strong TLR7 expression with HPV⁺ positivity remained. This confirms that the HPV⁺ and HPV⁻ BOTSCC behave differently regarding their immunological response, and thus are justly separated to different entities according to the latest WHO Head and Neck Pathology book (WHO Classification of Head and Neck tumors, Lyon 2017). Furthermore, due to the few events in this cohort among the HPV⁺ BOTSCC cases, we could not evaluate the correlation of the expression results with survival.

To conclude, in accordance with previous studies we could validate and confirm the presence of absent/weak TLR5 and medium/strong TLR7 expression in the HPV⁺ BOTSCC subtype, but not their correlation with clinical outcome. However, since TLR7 expression contrary to TLR5, was not correlated to CD8⁺ TIL counts, which itself being a very strong predictive marker, more studies on TLR7 as a prognostic marker are warranted.

Disclosure statement

No potential conflict of interest was reported by the authors.

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